Clinical Biochemistry 45 (2012) 1497–1502

Contents lists available at SciVerse ScienceDirect

Clinical Biochemistry

journal homepage: www.elsevier.com/locate/clinbiochem

Stabilization of cell-free RNA in blood samples using a new collection device

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ARTICLE INFO

Article history:
Received 26 March 2012
Received in revised form 31 May 2012
Accepted 4 July 2012
Available online 20 July 2012

Keywords:
RNA stability
Messenger RNA
Blood preservation

ABSTRACT

Objective: To investigate whether a new blood collection device stabilizes cell-free RNA (cfRNA) in blood post-phlebotomy when compared to collection using K_3EDTA tubes.

Design and methods: Blood samples were drawn from healthy donors into K_3EDTA tubes and Cell-Free RNA BCTs (BCTs) and stored at room temperature (20–25 °C). At specified time points (days 0–3), plasma was separated and cfRNA was extracted. Reverse transcription real-time PCR was used to quantify mRNA for c-fos, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and for 18S rRNA.

Results: Blood drawn into K_3EDTA tubes showed a steady increase in RNA concentration over 3 days of ex vivo incubation. Blood drawn into BCTs showed no statistically significant change in RNA copy number except for GAPDH on day 3.

Conclusions: The novel chemical cocktail contained in the new device allows for the stabilization of cfRNA in blood samples at room temperature, which potentially enhances the clinical utility of cfRNA.

Introduction

Since the discovery of cell-free nucleic acids (cfna) in peripheral blood [1], elevated cfna levels have been associated with certain disease conditions [2]. These findings have implicated cfna as diagnostic and prognostic indicators for disease pathology. The potential clinical use of cell-free RNA (cfRNA) was first demonstrated in patients with nasopharyngeal carcinoma [3] and malignant melanoma [4]. Later, tumor-associated mRNAs for telomerase components were detected in serum or plasma of patients with breast cancer [5], colorectal cancer, follicular lymphoma [6], and hepatocellular carcinoma [7]. Cytoke

breast c

ly, plasma samples from patients with advanced stage colorectal cancer contained detectable levels of cytokeratin 19 and carcinoembryonic antigen mRNA [9]. Wong et al. [10] has proposed that β-catenin mRNA might be used as a biomarker because of its presence in the plasma of patients with colorectal carcinoma and adenoma.

The occurrence of fetal cfRNA in maternal blood was initially reported in 2000 by Poon et al. [11] and subsequently other fetal/placental-specific cfRNAs were also found including human placental lactogen, the β-subunit of human chorionic gonadotropin, and corticotrophin-releasing hormone [12]. Additionally, elevated mRNA levels of corticotrophin-releasing hormone were found in women with pre-eclampsia [13]. Such discoveries provide opportunities for the development of non-invasive diagnostic and prognostic tests based on cfRNA. However, many pre-analytical variables remain that might impact the clinical utility of cfRNA that have yet to be studied in detail. The stability of cfRNA can be affected substantially during blood processing, shipping, and storage. It has been reported that cell-free β-actin mRNA levels in plasma decrease with time after blood collection likely due to in vitro RNA degradation [14]. Another study showed that cfRNA levels for both glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and beta-2-microglobulin increase with time after blood draw [15]. This increase in background RNA (bRNA) levels can hamper the detection of low abundance cfRNA targets. In order to use plasma cfRNA in clinically relevant assays

blood as well as maintenance of cfRNA integrity during sample manipulation. The aim of this study was to determine if a new blood collection device (Cell-Free RNA BCT) stabilizes cfRNA and prevents bRNA release.

Materials and methods

Recruitment of blood donors

Blood donors were recruited with informed consent from Streck Inc., Omaha, NE. Donors were both male and female, and presumed to be healthy.

Blood collection

Donor samples were drawn, using venipuncture, into two different blood collection tubes: a K_3EDTA blood collection tube (BD

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0009-9120
doi:10.1016/j.clinbiochem.2012.07.090

Vacutainer®, Becton Dickinson, Franklin Lakes, NJ) and a Cell-Free RNA™ BCT (BCT) (Streck, Inc. Omaha, NE). BCTs contain a novel stabilizing chemical cocktail as well as K_3EDTA as an anti-coagulant. Blood was mixed well immediately after the draw by inverting tubes 10 times each. BCTs and K_3EDTA draws were kept at room (20–25 °C) or refrigerated temperatures (2–8 °C) where indicated (note: the BCT instructions for use advise blood storage at 18–25 °C).

Sample processing

Plasma was separated from blood 2 h post collection (day 0) or after 1, 2, or 3 days as noted. To separate plasma, sample tubes were centrifuged at 300×*g* for 20 min at room temperature. The upper plasma layer was removed without disturbing the buffy coat and transferred to a new tube that was then centrifuged at 5000×*g* for 10 min to assure cell-free plasma. The clarified plasma was then transferred to a new tube for storage at −80 °C pending RNA extraction.

Cell-free RNA isolation from plasma

The cfRNA was extracted and purified from plasma using the QIAamp® Circulating Nucleic Acid Kit (Qiagen, Santa Clarita, CA). The manufacturer's recommended protocol was modified slightly by increasing the duration of the Proteinase K treatment at 60 °C from 30 min to 1 h (subsequent experiments showed no difference in RNA amplification between samples digested for 30 min or the modified 1 h [data not shown]). An on-column DNase treatment step was included to remove DNA and cfRNA was eluted in 30 μL nuclease-free water that was passed over the column twice.

Detection of RNase activity in plasma obtained from K_3EDTA and Cell-Free RNA BCT tubes

In preliminary experiments, plasma was separated from blood on day 0 from K_3EDTA tubes as described above and portion of plasma was treated with BCT chemical cocktail while another was left untreated. In a separate experiment, blood was drawn into BCT and K_3EDTA tubes, stored as whole blood at room temperature and plasma was separated as described above. RNase activity was measured using a commercially available RNase activity detection kit, RNaseAlert® Lab Test Kit (Life Technologies, Grand Island, NY) and the manufacturer's recommended protocol was followed.

Effect of chemical cocktail on cfRNA extraction and amplification

Blood from seven donors was drawn into K_3EDTA tubes, individual donors were pooled, plasma was separated, and aliquoted. Half of the aliquots were mixed with the chemical cocktail present in BCTs while the other half remained untreated. All samples were stored at room temperature. Total cfRNA was extracted from untreated and chemically treated plasma using lysis buffer without carrier RNA on days 0 and 3 and then total RNA was quantified using a NanoDrop spectrophotometer. For the experiments conducted to investigate the stability of β-actin and c-fos mRNAs, the same nucleic acid isolation kit was used without any modification to manufacturer's recommended protocol. Samples extracted with carrier RNA were analyzed using primers and probes for β-actin and c-fos to quantify β-actin and c-fos mRNA copy numbers as discussed below.

RT-qPCR

All primers were purchased from Integrated DNA Technologies (IDT) (Coralville, IA). All probes for TaqMan® assays were purchased from Applied Biosystems (Foster City, CA), except for β-actin, which was purchased from IDT. Primers and probe for the quantification by RT-qPCR of c-fos mRNA were prepared as previously described [16]. Primers and probe for RT-qPCR of GAPDH mRNA were also prepared as previously described [17]. The primers and probe used to quantify 18S rRNA by RT-qPCR were: 5′ (forward) primer was 5′-CGAATGTCTGCCCTATCAAC-3′; the 3′ (reverse) primer was 5′-GTTTCTCAGGCCCCTCTCC-3′; the probe was 5′-FAM GTAGGCACGGCGACTACCATCGAAA TAMRA-3′. To perform RT-qPCR, a TaqMan® RNA-to-C_T™ 1-Step Kit was purchased from Applied Biosystems (Foster City, CA). Plasmid DNA constructs were prepared by cloning a DNA fragment into Zero Blunt TOPO (Invitrogen, Carlsbad, CA); with each construct containing a single copy of human c-fos, GAPDH, or 18S rRNA. The resulting amplicons had lengths of 67 bp, 75 bp, and 105 bp, respectively. These plasmid constructs were used to plot the standard curves except for β-actin, which required a synthetic oligonucleotide (Ultramer™ Oligo) that was purchased from IDT. The primers and probe for β-actin were: 5′ (forward) primer; 5′-GAGAC CTTCAACACCCCAG-3′; 3′ (reverse) primer; 5′-ATCACGATGCCAGTGGT AC-3′, probe; 6-FAM-CCATGTACGTTGCTATCCAGGCTGT-3′TAMRA. This produced an 86 bp amplicon.

Blood cell stability

Blood from 8 donors was drawn into BCT and K_3EDTA tubes and plasma separated as described above. Evaluation of hemolysis was done by measuring plasma hemoglobin as previously described, using a Helios Beta UV–vis spectrophotometer (Thermo Spectronic, Rochester, NY) [18]. Whole Blood samples were evaluated for cell stability using an Abbott CELL-DYN® Sapphire® hematology analyzer (Santa Clara, CA).

Statistical analysis

Statistical analysis was carried out using the online free calculations version of GraphPad. (http://www.graphpad.com/quickcalcs/index.cfm). Paired Student's *t*-test was used and $p<0.05$ was considered statistically significant. Fold change calculations were determined as previously described [19].

Results

Effect of stabilization chemical cocktail on RNase activity

We tested if the proprietary chemicals present in BCTs inhibited RNase activity in blood, which would prevent the destruction of cfRNA during room temperature storage (Fig. 1A). The RNase activity of 5 μL of RNase A, as supplied with the kit, was used as a positive control. Addition of the chemical cocktail to 5 μL of RNase A, at the same concentration present in a 10 mL blood draw, resulted in a significant inhibition of RNase A activity ($p<0.001$). The RNase activity in 2 μL of plasma from blood drawn into K_3EDTA tubes showed an RNase activity comparable to the positive control. However, 2 μL of plasma from blood drawn into a BCT showed significant inhibition of RNase A activity ($p<0.001$).

Fig. 1B shows the effect of BCT chemical cocktail on RNase activity when blood was stored at room temperature over a 3 day period. Compared to plasma from blood stored in K_3EDTA tubes, BCT plasma showed an almost complete inhibition of RNase activity at all three time points ($p<0.001$ for all three points).

Effect of chemical cocktail on cfRNA extraction and amplification

We investigated whether the chemicals present in BCTs degrade or chemically modify cfRNA. Chemically treated and untreated plasmas were stored at room temperature over 3 days. Total cfRNA concentration measurement by spectrophotometry on day 0 and day 3 showed no statistically significant difference between the two treatment groups (Fig. 2A). Fig. 2B and C show the effects of the

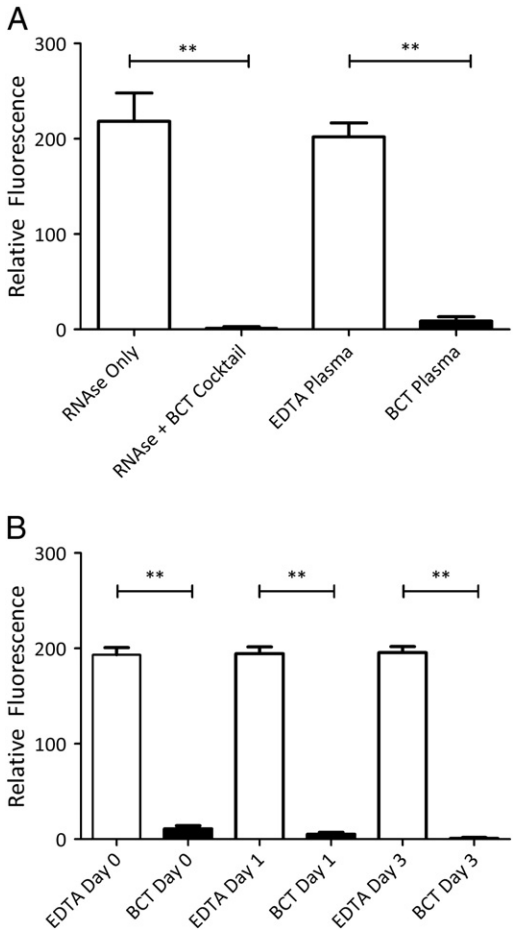

Fig. 1. RNase activity inhibition by BCT chemical cocktail. (A) Five microliters of RNase A (RNase Only) served as a positive control (RNaseAlert® Lab Test Kit). The chemical cocktail present in BCTs was then added to the reaction mixture of the positive control, which significantly reduced RNAse activity (RNase + BCT Cocktail). Plasma samples (2 μL) obtained from K_3EDTA (EDTA Plasma) and BCT (BCT Plasma) blood draws were tested for RNase activity following the manufacturer's recommended protocol. Plasma from blood drawn into BCT showed a significant reduction in RNAse activity compared to plasma from blood drawn into K_3EDTA. Error bars indicate SD, ($n=6$), $^{**}p<0.001$. (B) Whole blood was drawn into K_3EDTA (white bars) and BCTs (black bars) and stored at room temperature for 3 days to measure RNase activity over time. Aliquots of blood were removed on days 0, 1, and 3, plasma was separated, and 2 μL of the plasma was used to measure RNase activity. Error bars indicate SD, ($n=8$), $^{**}p<0.001$.

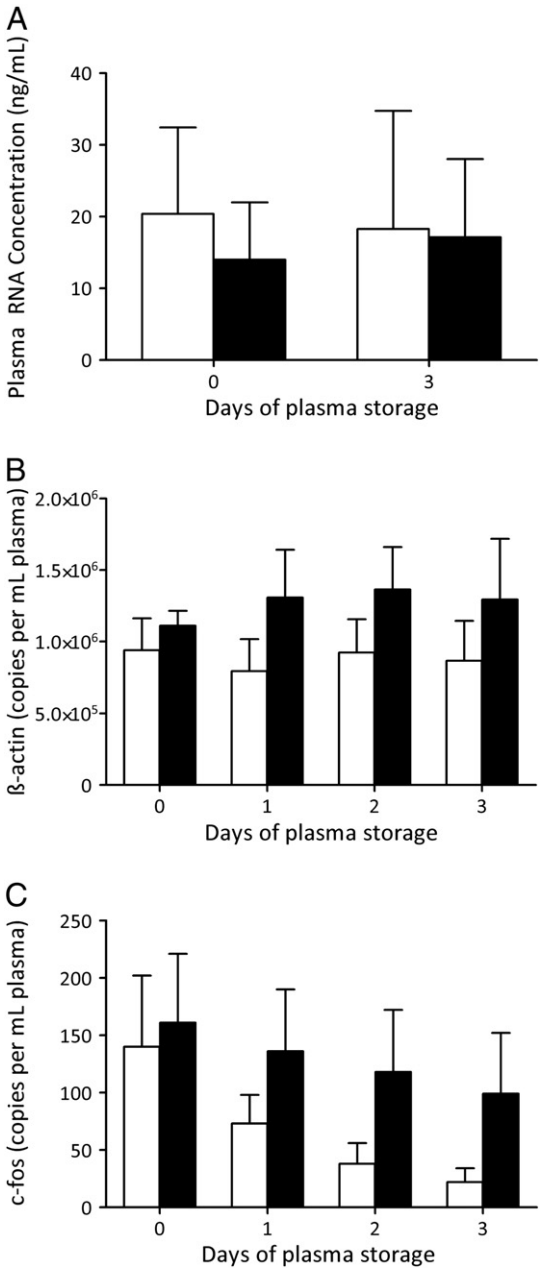

Fig. 2. Effect of chemical cocktail in BCTs on extraction, purification, and detection of RNA. For all experiments in Fig. 2, blood from seven donors was drawn into K_3EDTA tubes, the plasma was separated by centrifugation, and plasma was pooled before being aliquoted. To one group of plasma (black bars), chemicals were added that were equal to the concentration of BCT chemical cocktail present in a normal blood draw. The other group (white bars) was untreated and used as a control. Plasma was stored at room temperature. (A) To test the effects of the chemical cocktail on RNA extraction, plasma aliquots were removed from whole blood at days 0 and 3. Total RNA was extracted without carrier RNA and quantified using a NanoDrop spectrophotometer. Error bars indicates SD, ($n=7$). The effect of the chemical cocktail was also checked by detection of (B) β-actin and (C) c-fos mRNA by RT-qPCR. Aliquots of plasma were removed at days 0, 1, 2, and 3. Total RNA was extracted and β-actin and c-fos mRNA concentrations (copies/mL plasma) were determined by RT-qPCR. Throughout the three day storage, no significant decrease in β-actin mRNA was observed for untreated and chemically treated samples. Untreated samples showed a steady decrease in c-fos mRNA concentration over time in as compared to day 0 value. c-fos mRNA concentration in chemically treated samples showed no significant change up to day one, however there was moderate but statistically significant decline on days 2 and 3 (day 0 versus day 2; $p<0.001$; day 0 versus day 3; $p<0.001$). Error bars indicate SD, ($n=7$).

chemical cocktail on two individual mRNAs, β-actin and c-fos, respectively, using RT-qPCR to determine mRNA copy number. Fig. 2B illustrates that β-actin mRNA is stable in both treated and untreated plasma over a 3 day period at room temperature and showed no statistically significant decrease in β-actin amplification. However, c-fos mRNA was considerably more labile under the same conditions (Fig. 2C). Untreated plasma c-fos mRNA showed a statistically significant decrease on days 1, 2, and 3 as compared to the initial time point. There was no statistically significant decrease in chemically treated plasma c-fos mRNA after 1 day of storage, and a small but statistically significant decrease was observed on days 2 ($p=0.0082$) and 3 ($p=0.0062$) as compared to day 0.

Stabilization of cfRNA

We evaluated the effects of BCTs versus K_3EDTA on the stabilization of cfRNA levels in whole blood draws stored over 3 days at room temperature. RT-qPCR was run to quantify cfRNA using gene-specific assays for GAPDH, c-fos, and 18S RNA. Fig. 3A illustrates the changes observed in GAPDH mRNA levels over the three day storage period. When blood was drawn and stored in K_3EDTA tubes, statistically significant changes occurred over the storage period ($p=0.0017$, 0.0003,

and 0.0067 for days 1, 2 and 3, respectively). In blood samples in BCTs, measurement of GAPDH copy number did not show statistically significant changes after 2 days of storage, as compared to day 0 ($p=$ 0.2753 and 0.3662, respectively) but day 3 did show a statistically significant difference when compared to the initial data point ($p=$ 0.0012). However, when fold change calculations were performed, the increase in GAPDH on day 3 for BCT was only 1.5 fold versus an increase of 22 fold in K_3EDTA.

Fig. 3B shows the changes in c-fos levels over a three day storage period. When blood was drawn into K_3EDTA and stored, there was a steady increase in c-fos copy number detected over the three day experiment as compared to the day 0 copy number ($p=0.1168$, 0.0526, and <0.0001, respectively), which was a statistically significant increase on day 3. The BCT draws, however, showed no statistically significant change over the course of the study ($p=0.3289$, 0.5448, and 0.4531, respectively).

Fig. 3C shows the results for 18S rRNA over the three day storage period. When compared to the day 0 copy number, 18S cfRNA from blood drawn into BCTs show no statistically significant increases on days 1, 2, or 3 ($p=0.4400$, 0.5672, and 0.8719, respectively). In contrast, the samples from blood drawn into K_3EDTA show a statistically significant increase in 18S rRNA copy number over the course of the same storage period ($p=0.0002$, 0.0027, and 0.0008, respectively).

Finally, when the cfRNA stabilization studies were repeated with refrigerated instead of room temperature storage, the changes observed were statistically similar. This indicated that refrigeration of blood samples drawn into K_3EDTA does not sufficiently mitigate the changes from bRNA leakage or cfRNA degradation (data not shown).

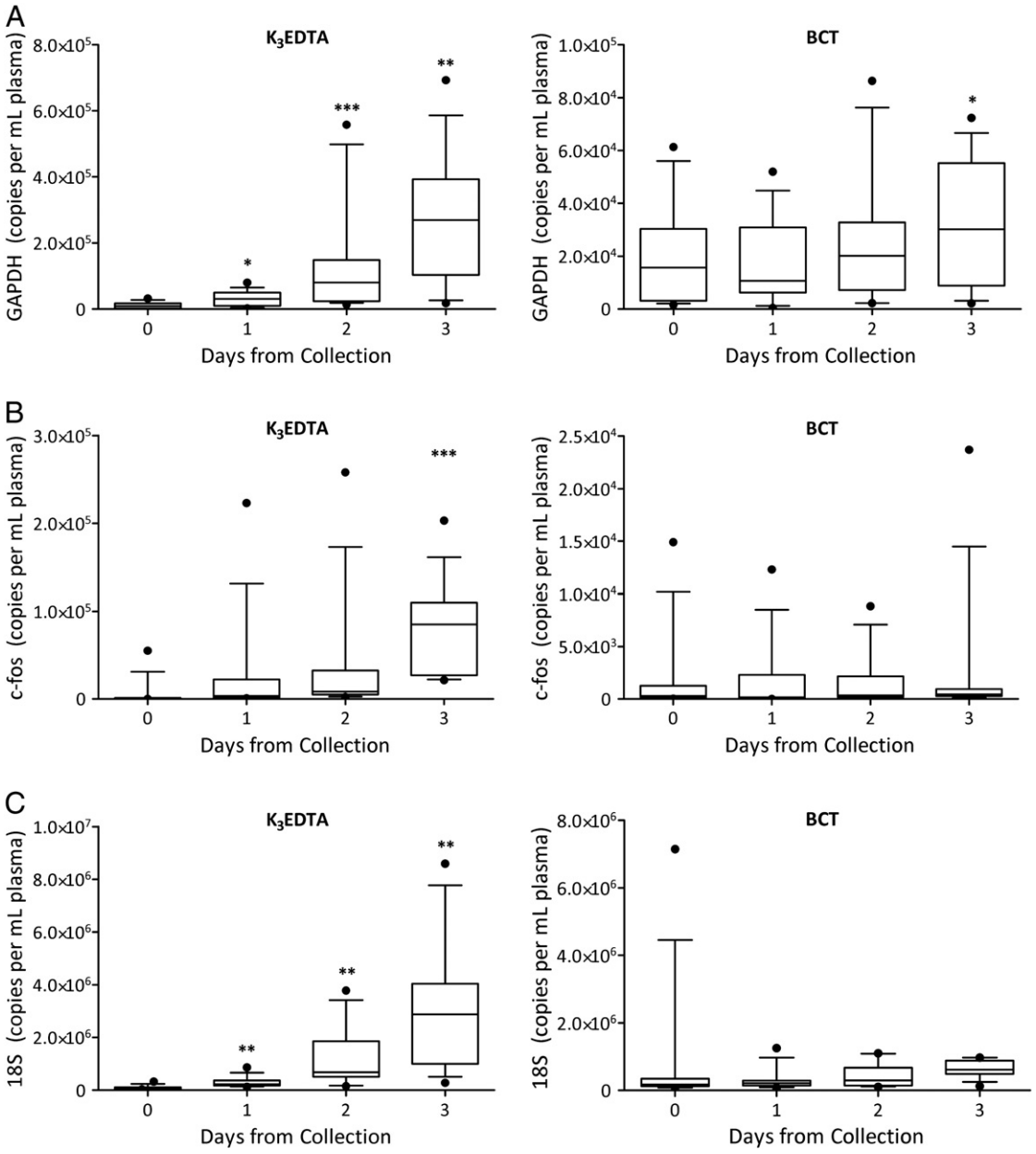

Fig. 3. Effect of ex vivo incubation of blood samples on plasma RNA concentration in K_3EDTA tubes or BCTs. Blood from fifteen donors were drawn into K_3EDTA tubes and BCTs and stored at room temperature. Aliquots were removed on days 0, 1, 2, and 3, plasma RNA was isolated, and RNA for (A) GAPDH, (B) c-fos, and (C) 18S rRNA were quantified by RT-qPCR. Over time, statistically significant changes in the copy numbers of GAPDH, c-fos, and 18S rRNA were observed in K_3EDTA samples while there were no significant copy number changes in blood drawn into BCTs except for GAPDH day 3 time point where there was a slight (1.4-fold) but statistically significant increase. The line inside of the boxes indicates the median value. The limits of the boxes represent the 75th and 25th percentiles. The upper and lower error bars indicate the 10th and 90th percentiles. The dots indicate maximum and minimum values. $^*p<0.05$, $^{**}p<0.01$, $^{***}p\leq0.001$ (GAPDH $n=15$, c-fos $n=14$, and 18S $n=13$).

Blood cell stability

We evaluated hemolysis by measuring plasma hemoglobin concentration. Mean hemoglobin concentrations in K_3EDTA tubes on days 0, 1, and 3 were 0.029, 0.022, and 0.029 g/L, respectively, while BCT had 0.034, 0.021, and 0.023 g/L on days 0, 1 and 3, respectively. There was neither significant hemolysis nor statistical change in plasma hemoglobin concentration observed during the three day room temperature storage for both K_3EDTA tubes and BCT, suggesting that the integrity of red cells was maintained. Analysis of whole blood samples on the Abbott CELL-DYN Sapphire hematology analyzer confirmed this finding. Both red cell counts and histograms remained stable through the three day testing period. However, blood drawn in K_3EDTA tubes started to show diffused white cell scattergrams after 1 day of room temperature storage, which indicates cell damage and degradation. Conversely, scattergrams of white cells in BCT indicated that the cells were well preserved and stable for up to 3 days (data not shown).

Discussion

Circulating cfRNA in blood has potential value in non-invasive diagnosis and prognosis. Two major factors appear to limit the effective use of cfRNA in clinical testing: (1) the masking of true cfRNA targets due to bRNA release and (2) the degradation of cfRNA due to plasma nucleases or sample handling. The high concentration of RNases in plasma has been established previously [20]. With such high levels of RNase activity in plasma, it is surprising that cfRNA can be detected. Some studies [21–23] have proposed that cfRNA might be contained in apoptotic bodies, whose sealed membranes provide cfRNA protection from RNase degradation. Others [24] suggest that plasma cfRNA exists in two forms, contending that a substantial proportion exists as a particle associated species that is protected from degradation while the remainder is present in a non-particle associated form that is labile and susceptible to degradation.

Holford et al. [14] have found that β-actin cfRNA concentrations in plasma decrease over time when stored at 4 °C, which they attributed to RNase-based degradation. Therefore, it is important to rapidly inhibit plasma RNases in blood samples to assure that cfRNA integrity is preserved. As shown in Fig. 1, if chemicals present in BCTs were mixed directly with RNase, the enzyme activity was inhibited. We have further shown that plasma from blood drawn into the new device has little remaining RNase activity but plasma from blood drawn into K_3EDTA showed no decrease in RNase activity. This suggests that one of the two main limiting factors for the clinical utilization of cfRNA can be mitigated by using BCTs through the rapid inactivation of plasma RNases.

We wanted to determine if the chemical cocktail present in BCT caused a decrease in RNA isolation or amplification (Fig. 2). To this end, we compared cfRNA yields isolated from untreated plasma to yields from the same plasma after treatment with the collection device's chemical cocktail (Fig. 2A). When compared to day 0, no statistically significant decreases in total cfRNA yields were observed between the treated group and the untreated group over 3 days of storage at room temperature. This indicates that chemicals present in the BCTs had no adverse effect on cfRNA isolation from blood plasma.

We investigated the effect of chemical cocktail on the amplifiability of plasma cfRNA using RT-qPCR of β-actin and c-fos as targets and found no statistically significant decrease in treated plasma compared to untreated plasma (Fig. 2B and C). Holford et al. [14] however, studied the stability of plasma β-actin mRNA in blood stored at 4 °C over 5 h and had shown a rapid decrease in β-actin mRNA concentration within the first hour post phlebotomy. After this initial change, β-actin mRNA was then relatively stable for up to 5 h. This rapid change is not reflected in our β-actin measurements, which we suggest may be due to the clearance of the non-particle associated cfRNA by the action of plasma RNases. In our experiments described in Fig. 2, 1 to 2 h elapsed during the preparation of untreated and treated plasmas. Any processing delay may allow the degradation of non-particle associated cfRNA in our samples by plasma RNases, leaving only particle associated RNase resistant cfRNA. This may explain the discrepancy between our results and the results published by Holford et al.

Comparison of c-fos mRNA concentrations in untreated and chemically treated groups also shows that the chemical cocktail had no adverse effect on the amplifiability of RNA (Fig. 2C). The treated group had higher mRNA copy numbers as compared to the untreated group at all time points. However, the stability of proto-oncogene c-fos mRNA in plasma at room temperature differed from the housekeeping gene β-actin mRNA. There was a significant decrease in c-fos mRNA concentration in the untreated group over time. We speculate that this difference may be due to the inherent instability of c-fos mRNA at room temperature. In the chemically treated group, c-fos mRNA was relatively stable compared to the untreated group and showed decreases on days 2 and 3 (0.7-fold and 0.6-fold, respectively). We suggest that the improved stability compared to untreated plasma is conferred by the BCT chemical cocktail.

Salway et al. [15] found that plasma GAPDH mRNA concentrations increase with time in whole blood samples. Our experiments on blood cell stability have shown that erythrocytes are stable in K_3EDTA and BCT during 3 days of incubation at room temperature while white blood cells were stable only in BCT. In the K_3EDTA collection device, white blood cells degrade markedly after 24 h of storage at room temperature. Therefore, we suggest that the increase in GAPDH mRNA concentration observed by Salway et al. is caused by the release of bRNA during cell degradation, most likely that of white blood cells. In our RT-qPCR experiments of GAPDH, c-fos, and 18S cfRNA, ex vivo room temperature storage of blood samples drawn into K_3EDTA tubes resulted in statistically significant increases in mRNA concentration. Detection of the same genes did not significantly change in plasma isolated from whole blood that had been stabilized in BCTs except for GAPDH on day 3, which showed a minor 1.5-fold increase. This suggests that the cocktail present in the new blood collection device minimized non-specific bRNA release into plasma during sample processing and storage, which could have masked the presence of cfRNA and limit its use as a biomarker.

In order to minimize cfRNA degradation and prevent increases in bRNA, present protocols dictate that whole blood should be centrifuged immediately post-phlebotomy and the plasma processed. However, in situations where testing is performed at a location other than the blood draw site, this procedure becomes even more challenging due to the requirement for cryopreservation after plasma separation. This necessitates that phlebotomy sites have the equipment for centrifugation and cryopreservation as well as a trained staff. Use of the new device could overcome these burdens, since the novel chemical cocktail significantly inhibits plasma RNase activity and prevents the non-specific release of bRNA. Storage of cfRNA samples in the original draw tube at room temperature for up to 3 days is now a viable option. This stabilization technology would permit smaller labs to collect multiple samples for optimized single processing events or allow for transfer of stabilized samples to a more competent or better equipped core-like facility. The BCT could provide convenience for shipment to a centralized facility for sample processing and analysis. All of these situations require the additional flexibility and utility in cfRNA analysis that would be provided by the novel device, in research and clinical settings.

Acknowledgments

We the authors gratefully acknowledge the technical assistance provided by Gary Krzyzanowski for this study. We also thank Dr. Chris Connelly for his valuable advice and critical reading of this manuscript.

References

[1] Mandel P, Métais P. Les acides nucléiques du plasma sanguine chez l'homme. C R Acad Sci Paris 1948;142:241-3.
[2] Tan EM, Schur PH, Carr RI, Kunkel HG. Deoxyribonucleic acid (DNA) and antibodies to DNA in the serum of patients with systemic lupus erythematosus. J Clin Invest 1966;45:1732-40.
[3] Lo KW, Lo YM, Leung SF, Tsang YS, Chan LYS, Johnson PJ, et al. Analysis of cell-free Epstein–Barr virus associated RNA in the plasma of patients with nasopharyngeal carcinoma. Clin Chem 1999;45:1292-4.
[4] Kopreski MS, Benko FA, Kwak LW, Gocke CD. Detection of tumor messenger RNA in the serum of patients with malignant melanoma. Clin Cancer Res 1999;5:1961-5.
[5] Chen XQ, Bonnefoi FA, Pelte MF, Lyautey J, Lederry C, Movarekhi S, et al. Telomerase RNA as a detection marker in the serum of breast cancer patients. Clin Cancer Res 2000;6:3823-6.
[6] Dasi F, Lledo S, Garcia-Granero E, Ripoll R, Marugán M, Tormo M, et al. Real-time quantification in plasma of human telomerase reverse transcriptase (hTERT) mRNA: a simple blood test to monitor disease in cancer patients. Lab Invest 2001;81:767-9.
[7] Miura N, Shiota G, Nakagawa T, Maeda Y, Sano A, Marumoto A, et al. Sensitive detection of human telomerase reverse transcriptase mRNA in the serum of patients with hepatocellular carcinoma. Oncology 2003;64:430-4.
[8] Silva JM, Dominguez G, Silva J, Garcia JM, Sanchez A, Rodriguez O, et al. Detection of epithelial messenger RNA in the plasma of breast cancer patients is associated with poor prognosis tumor characteristics. Clin Cancer Res 2001;7:2821-5.
[9] Silva JM, Rodriguez R, Garcia JM, Muñoz C, Silva J, Dominguez G, et al. Detection of epithelial tumor RNA in the plasma of colon cancer patients is associated with advanced stages and circulating tumor cells. Gut 2002;50:530-4.
[10] Wong SC, Lo SF, Cheung MT, Ng KO, Tse CW, Lai BS, et al. Quantification of plasma beta-catenin mRNA in colorectal cancer and adenoma patients. Clin Cancer Res 2004;10:1613-7.
[11] Poon LLM, Leung TN, Lau TK, Lo DYM. Presence of fetal RNA in maternal plasma. Clin Chem 2000;46:18321834.
[12] Ng EK, Tsui NB, Lau TK, Leung TN, Chiu RWK, Panesar NS, et al. From the cover: mRNA of placental origin is readily detectable in maternal plasma. Proc Natl Acad Sci U S A 2003;100:4748-53.
[13] Ng EK, Leung TN, Tsui NB, Lau TK, Panesar NS, Chiu WRK, et al. The concentration of circulating corticotrophin-releasing hormone mRNA in maternal plasma is increased in preeclampsia. Clin Chem 2003;49:727-31.
[14] Holford NC, Sandhu HS, Thakkar H, Butt AN, Swaminathan R. Stability of β-actin mRNA in plasma. Ann N Y Acad Sci 2008;1137:108-11.
[15] Salway F, Day PJR, Ollier WER, Peakman TC. Levels of 5′ RNA tags in plasma and buffy coat from EDTA blood increase with time. Int J Epidemiol 2008;37:i11-5.
[16] Gan L, Doroudi R, Hägg U, Johansson AN, Selin-Sjögren L, Jern S. Differential immediate-early gene response to shear stress and intraluminal pressure in intact human conduit vessels. FEBS Lett 2000;477:89-94.
[17] Cohrs RJ, Gilden DH. Prevalence and abundance of latently transcribed varicella-zoster virus genes in human ganglia. J Virol 2007;81:2950-6.
[18] Fairbanks VF, Ziesmer SC, O'Brien PC. Methods for measuring plasma hemoglobin in micromolar concentration compared. Clin Chem 1992;38:132-40.
[19] Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci U S A 2001;98(9):5116-21.
[20] Herriott RM, Connolly JH, Gupta S. Blood nucleases and infectious viral nucleic acids. Nature 1961;4767:817-20.
[21] Tsui NB, Ng EK, Lo YM. Stability of endogenous and added RNA in blood specimens, serum, and plasma. Clin Chem 2002;48:1647-53.
[22] Halicka HD, Bender E, Darzynkiewicz Z. Segregation of RNA and separate packaging of DNA and RNA in apoptotic bodies during apoptosis. Exp Cell Res 2000;260: 248-56.
[23] Hasselmann DO, Rappl G, Tilgen W, Reinhold U. Extracellular tyrosinase mRNA within apoptotic bodies is protected from degradation in human serum. Clin Chem 2001;47:1488-9.
[24] Ng EK, Tsui NB, Lam NY, Chiu RWK, Yu SCH, Wong SCC, et al. Presence of filterable and non-filterable mRNA in the plasma of cancer patients and healthy individuals. Clin Chem 2002;48:1212-7.